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PLATELET ABNORMALITIES IN IDIOPATHIC MYELOFIBROSIS: FUNCTIONAL, BIOCHEMICAL AND IMMUNOMORPHOLOGICAL CORRELATIONS

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ABSTRACT

Background. **An extensive study of platelet function was performed on 18 consecutive patients affected by idiopathic myelofibrosis (IM).**

Materials and methods. **Clinical hematological and morphofunctional parameters were studied in IM patients and control subjects. Platelet tests, ultrastructural data, immunocytochemical von Willebrand factor detection, freeze fracturing results and free cytosolic calcium level were evaluated.**

Results. **Bleeding time was frequently found to be prolonged, but it never reached levels which could give any cause for concern. Aggregation by ADP, collagen and epinephrine was always altered, sometimes profoundly; on the contrary, agglutination by ristocetin was almost always normal, albeit occasionally increased. Plasma β-TG and PF4 levels were found to be elevated in 11 and 12 patients, respectively. This indicated an abnormal release from platelet α-granules. Depletion of α-granules was also confirmed by the intraplatelet von Willebrand factor (vWF) labelling with colloidal gold particles bound to polyclonal antibodies against human vWF. In fact: 1) the number of positive α-granules/μm^2 and per single platelet was reduced; 2) the intensity of the immunocytochemical reaction for single positive α-granules and for each platelet was significantly reduced. Freeze-fracturing studies showed an increase in the number of intra-membrane particles (IMP) on the P face of the platelet membrane with respect to normal platelets preincubated with ADP. However, no differences in their distribution or diameter were observed. High concentrations of free cytosolic calcium were always found and Ca^{++}ATPase activity was increased. Conversely, Na^+/K^+ ATPase activity was always reduced.**

Conclusions. **We can hypothesize that the platelet membrane is altered in IM, resulting in facilitated activation, even by subliminal stimuli, and that this continuous platelet activation ultimately leads to α-granule depletion.**

Key words: platelet, idiopathic myelofibrosis, von Willebrand factor, plasma membrane

Bleeding and thrombosis are complications which frequently arise in patients affected by *chronic myeloproliferative disorders* (CMDs), particularly in patients with *essential thrombocythemia* (ET) and *polycythemia vera* (PV), but also in those with *idiopathic myelofibrosis* (IM).[1,2] The pathophysiology of hemostatic defects in CMDs is rather complex and, to date, has only been partially elucidated.

IM is associated with numerous platelet morphological abnormalities, which can be visualized by either light (anisocytosis, *blue platelet*)

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or electron microscopy (abnormal granules, features of immaturity).[3] A modified membrane glycoprotein pattern has also been observed,[4, 5] along with altered intraplatelet calcium metabolism, which plays a pivotal role in platelet activation.[6]

Several coagulative[7] and functional platelet abnormalities (bleeding time, adhesiveness, aggregation and release action) have also been described in IM[8] as well as in other CMDs.[9]

The most frequent platelet abnormalities are: reduced aggregatory response to epinephrine,[9] PGD_2 receptor deficiency,[10] storage pool defects,[11] increase in TGF-β content,[12] alterations in arachidonic acid metabolism.[13]

Low intraplatelet and high plasma levels of β-TG and PF4[14] have also been observed, suggesting *in vivo* platelet activation leading to α-granule depletion. Von Willebrand Factor (vWF) is also present in the α-granules and is released upon ADP, collagen and thrombin stimulation.[15] Low platelet levels of vWF have been found in both ET and PV,[15] while either normal or elevated values are seen in CML.[8]

In order to gain further insight into this problem, functional, ultrastructural and biochemical studies were carried out on 18 consecutive patients affected by IM; our aim was to evaluate whether platelet malformation and/or low-grade, *abnormal in vivo activation* are present in this pathology.

Materials and methods

Patients

Eighteen patients, 12 males and 6 females aged 41-77 years (mean 63.9 yrs), affected by idiopathic myelofibrosis (IM) were studied. Diagnosis of IM was made according to standard criteria, and histological staging was evaluated according to Lewis.[16] Clinical and hematological patient data are shown in Table 1. Mean hemoglobin value, as well as leukocyte and platelet numbers were highly variable.

Bleeding time was determined according to Ivy's method,[17] using a Surgicat device (Ortho Diagnostic Systems, Milan, Italy).

Platelet tests

Platelets were counted with a Toa Sysmex series E5000 electronic particle counter (Toa Medical Electronics, Kobe, Japan) on whole blood collected in EDTA (1 mg/mL). All the blood samples for platelet tests were taken with a large-bore needle so as to avoid blood stasis.

β-TG and PF4 levels were evaluated using commercially available ELISA kits (Boehringer Mannheim, Germany). Venous blood was collected in vacutainer tubes pre-cooled to 0-4°C, containing a mixture of 0.1 mL EDTA (10%), 0.1 mL theophylline (5.4 mg/mL) and prostaglandin E1 (1 ng/mL) as anticoagulant and antiplatelet reagents, respectively. The tubes were immediately transferred to an ice bath and left for 30' before centrifugation (1500 g for 30' at 4°C). The platelet-poor plasma (PPP) was immediately frozen and stored at –20°C until processing. The normal range was calculated on a control population (30 apparently healthy subjects aged 22-70 years, mean 65 yrs).

Aggregation was evaluated according to the Born method[18] using an Aggricorder PA-3210 aggregometer (Menarini Diagnostic, Florence, Italy). Venous blood was collected in 3.8% sodium citrate (9 vol:1 vol) and platelet-rich plasma (PRP) was obtained by centrifugation for 10' at 200 g. The platelet concentration of PRP was adjusted to 300×10^9/L by dilution with autologous PPP (centrifugation at 1100 g for 10'). The final concentration of the platelet agonists was: collagen 8 μg/mL, ADP 4 μM, epinephrine 10 μM (Simmelweiss, Mascia Brunelli, Milan, Italy), ristocetin (Menarini Diagnostic) 1.5 mg/mL. The parameters studied for collagen included initial latency (IL) of response (expressed in seconds), maximum width (MW)

Table 1. Clinical and hematological data from 18 patients affected by IM.

	M ± SD	range
Age (years)	63.9 10.9	41-77
Duration of disease (months)	71.3 60.5	1-192
Hb (g/dL)	10.9 2.5	5.9-14.6
WBC (x10^9/L)	13.6 22.5	1.5-100
Plt (x10^9/L)	248 129	39-455

	Spleen size					Histol. phase			
	S	I	II	III	IV	I	II	III	IV
# of patients	1	2	4	4	7	2	5	7	4

Scoring system for spleen size: S = splenectomy; I = spleen hardly palpable under costal margin; II = intermediate between I and III; III = spleen reaching the transumbilical line; IV = spleen exceeding the transumbilical line. See text for histological phase.

of response (expressed as the percentage of transmittance) and latency to 50% (L/50) maximum amplitude of response (expressed in seconds).

Ultrastructural studies

We also studied platelets from IM patients, using those of 2 normal subjects as controls. The platelet pellets employed for these ultrastructural observations were obtained in the following manner: venous blood (10 mL) was drawn from a cubital vein, mixed with 0.3 mL disodium EDTA 5% and centrifuged at 150 g for 15'. The PRP was collected and added to an equal volume of an antiaggregant buffer solution (Tris-HCl 10 mM, EDTA 1 mM, glucose 5 mM, NaCl 150 mM), pH 7.2, and subsequently centrifuged 3 times at 1500g for 15' in order to separate the platelets. Three pellets were prepared for each patient, fixed in 1% glutaraldehyde plus 0.1 M cacodylate buffer solution for 30', and processed for observation in a transmission electron microscope (TEM) and for freeze fracturing. The platelet pellets were dehydrated in increasing concentrations of ethyl alcohol and propylene oxide, impregnated with araldite at decreasing concentrations of propylene oxide, and finally embedded in araldite at 60°C for 2-3 days. Glycolmethacrylate (GMA) embedding was performed according to Leduc and Bernhard.[19] Briefly, the pellets were impregnated in increasing concentrations of aqueous GMA solutions, then embedded in a mixture containing GMA, butylmethacrylate and benzoyl peroxide at 4°C for 5 days under UV light.

Platelet von Willebrand factor evaluation

Ultrathin sections (600-700 Å thick) were obtained from GMA-embedded samples with a Reichert Ultracut E ultramicrotome and then transferred onto nickel grids for immunocytochemical tests according to Cramer et al.[20]

Briefly, rabbit anti-human vWF polyclonal antiserum (Dakopatts, Copenhagen, Denmark) was added to the slices and the reaction was then developed with goat antiserum against rabbit IgG bound to 10 nm colloidal gold particles (Janssen, Geel, Belgium). Tests omitting incubation with vWF antisera always proved to be negative and served as controls. The grids, shadowed with uranyl acetate and lead citrate, were studied at 18,000× magnification with a Philips EM 301 Electron Microscope (Philips, Eindoven, The Netherlands) operating at 80 Kv.

Platelet area, number of positive α-granules per μm^2 and per platelet, and number of colloidal gold particles per each positive α-granule were measured on the final photos using an IPS-Kontron image analyzer (IBAS by Zeiss, Oberkochen, Germany). Only α-granules with a clearly visible section on the photos were taken into consideration.

Freeze-fracturing

Platelet pellets, previously fixed in 1% glutaraldehyde and washed in 0.1 M cacodylate buffer, were cryoprotected with a solution of 27% glycerol in 0.1 M sodium cacodylate for 60'. The samples, reduced to small cubes (0.5 nanometers), were mounted on gold discs, rapidly frozen in freon 22 and liquid nitrogen, and fractured using a Balzers 301 (Balzer Union Aktiengesellschaff, Furstentum, Liechtenstein) device at –113°C, 2×10^{-6} torr, without etching.

Fracture surfaces were shadowed with 22 Å carbon-platinum replicas at 45°C and stabilized by carbon deposition at 60 mA for 50 seconds. Replicas were then dipped into sodium hypochlorite, washed in bidistilled water and mounted on copper-rhodium grids (300 mesh). Fracture surface replicas were studied by means of a Philips EM 301 electron microscope at 80Kv. For image analysis, we chose only the flat zones of the platelet membrane, excluding the surface connecting system (SCS) zones and all other irregularities. The photos, at a final magnification of 136,000×, were processed by an IPS-Kontron image analyzer. *Intramembrane particles* (IMP) appeared as dark, roundish particles surrounded by a lighter shadow.

We calculated the IMP density and diameter (perpendicular to the direction of the shadow) in randomly chosen areas. *Distribution factor* (FAC-V) was calculated as the ratio between the variance and the mean number of IMPs per area. FAC-V is equal to 1 when the particles have a random distribution, less than 1 if they are not randomly distributed and form a regular pattern, and greater than 1 if they form clusters.[21]

Three normal subjects whose samples were prepared after incubation of platelets with the same agonists used in the aggregation tests served as controls.

No platelet aggregation was observed at blood collection in EDTA. Platelet suspensions were

processed similarly to the other unstimulated samples.

Platelet aggregates

Platelet aggregates from both normal subjects and patients were obtained in vitro from PRP after adding collagen (8 μg/mL) or ristocetin (1.5 mg/mL). Aggregates were isolated from plasma, fixed in 1% glutaraldehyde in 0.1 cacodylate buffer for 30', post-fixed in OsO_4 and dehydrated in increasing concentrations of ethyl alcohol and propylene oxide. After being impregnated in resin and propylene oxide, the samples were embedded in araldite at 60°C for 2 days.

Cytosolic-free calcium concentrations

The level of ionized calcium in blood platelets was measured according to the method of Rao.[22] Blood was drawn and immediately mixed with acid citrate dextrose (ACD: 0.1 M citrate, 7 M citric acid, 140 M dextrose, pH 6.5) in a ratio of 9:1 (v/v). Platelet-rich plasma was obtained after having centrifuged whole blood for 20' at 140 g at room temperature. Platelet count was adjusted to 300×10^9/L by adding platelet-poor plasma.

The cells were then washed twice in an anti-aggregant buffer (10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 5 mM glucose, pH 7.3). To load the calcium-sensitive probe Fura-2, platelets were incubated at 37°C for 45' with 1 mM Fura-2-acetoxymethylester (Fura-2 AM) in a solution containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose, pH 7.4. Cells were then washed again in the same solution in order to remove excess dye.

Intracellular Ca^{++} levels were determined by using a Perkin Elmer MPF-66 (Perkin Elmer, Norwalk, USA) fluorescence spectrophotometer at 37°C according to the Grynkiewicz method.[20]

Ca^{++} ATPase and Na^+/K^+ ATPase assay

In order to obtain platelet membranes, blood was drawn in ACD anticoagulant and PRP was separated by centrifugation at 140 g for 20' at room temperature. The platelets were then washed in a buffer solution containing 8 mM NaH_2PO_4, 2 mM NaH_2PO_4, 10 mM EDTA, 5 mM KCl, 135 mM NaCl, pH 7.0, and pelleted by means of centrifugation at 3000 g for 30'.

The cells were then lysed by ultrasonication

Table 2. Results of functional platelet tests in 18 patients affected by IM. Note the high percentage of IM patients with high β-TG and PF-4 serum levels together with alterations of platelets aggregation. Bleeding time, although frequently prolonged, never reached levels which gave reason for concern.

Tests	normal values	patient data mean±SD	range	n		%
Bleeding time	3-7'	7'42"±1'25"	5'-9'30"	12/18		66.6
Platelets (x 10^9/L)	150-400	243±129 3/18	39-455 (> 400)	6/18 16.7	(< 150)	33.3
Aggregation						
- collagen						
IL (sec)	34-70	175±88.3	52-300	16/18		88.8
L/50 (sec)	45-135	210±108	5.6-360	14/18		77.7
MW (%)	> 75	55.1±40.8	5-140	11/18		61.1
- epinephrine-MW (%)	> 62	7.8±8.9	1-36	15/18		94.4
- ADP-MW (%)	> 65	16.4±15	1-64	16/18		88.8
- ristocetin-MW (%)	50-86	87.1±16	53-99	0/18	(< 50)	0
				11/18	(> 86)	61.1
β-TG (UI/L)	< 50	72±42	15-142	11/18		61.1
PF-4 (UI/L)	< 50	17±11	4-48	12/18		66.6

IL = initial latency; L/50 = latency to 50%; MW = maximum width.

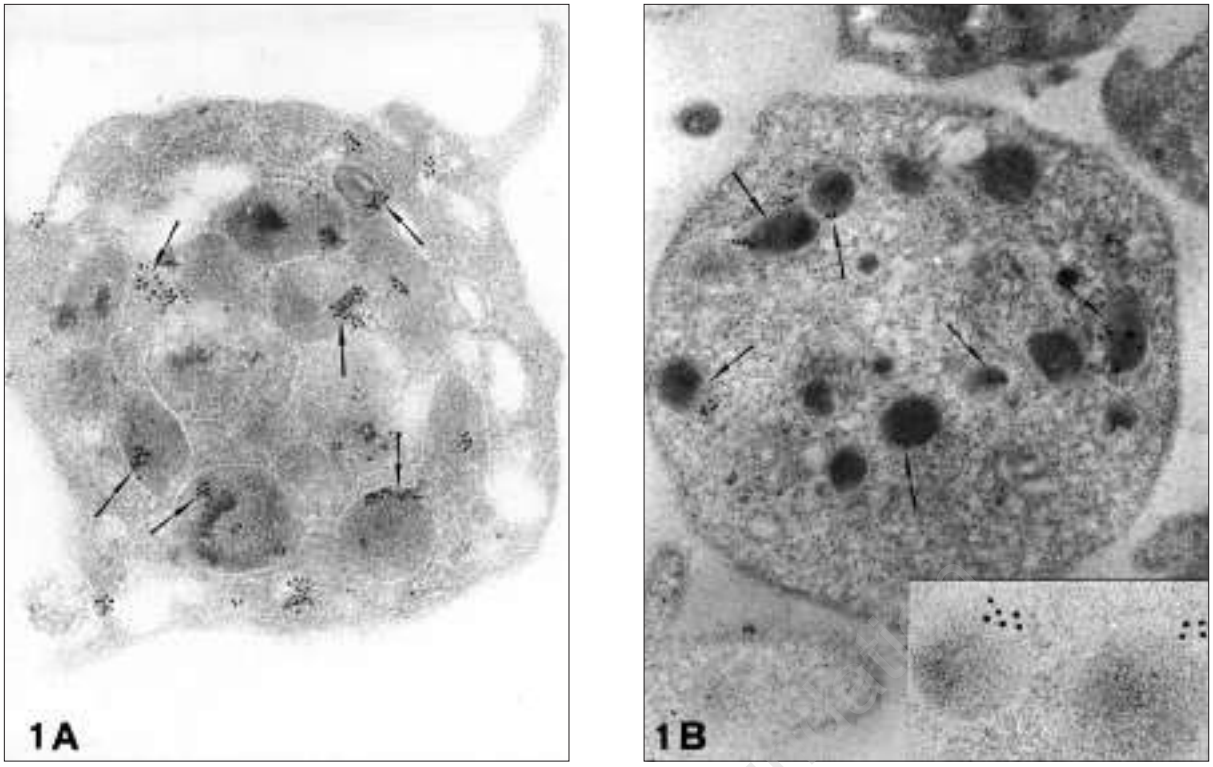

Figure 1. Immunocytochemical reaction of anti-vWF in the α-granules of platelets from a normal subject (A) and a patient with IM (B). Reduction of immunogold particles (⟶) in the platelets of patients with IM should be noted (B). Magnification 38,000x (Insert 95,000x).

and the platelet membrane fraction, corresponding to the plasma membrane, was isolated as described by Enouf et al.[24] Ca^{++}ATPase activity was then determined according to the method of Davis et al.[25] by measuring the inorganic phosphate (Pi) hydrolyzed from 1 mM Na_2 ATP at 37°C in the presence and absence of 0.15 mM Ca^{++}.

The ATPase activity determined in the absence of Ca^{++} was subtracted from total ATPase activity in order to calculate Ca^{++}ATPase activity. Na^+/K^+ ATPase activity was determined by using a modified form of the Kitao method.[26] This ATPase activity was assayed by incubating platelet membranes at 37°C in 1 mL of medium ($MgCl_2$ 5 mM, NaCl 140 mM, KCl 14 mM, in Tris-HCl 40 mM pH 7.7).

The ATPase reaction was initiated by adding 3 mM Na-ATP and stopped 20' later by adding 1 ml trichloroacetic acid 15%. ATPase activity, assayed in the presence of 10 mM ouabain, was subtracted from total Mg^{++}-dependent ATPase activity in order to calculate the ouabain-sensitive Na^+/K^+ ATPase activity.

Ca^{++}ATPase activity results are expressed as μmol Pi/(mg membrane proteins × 90'), while the Na^+/K^+ ATPase activity results are expressed as μmol Pi/(mg membrane proteins × 60').

Statistical analysis

Statistical evaluation of biochemical data was carried out using Student's t-test for unpaired data. Student's t-test, modified according to Bonferroni for multiple comparisons, was used for evaluation of the ultrastructural studies.

Results

Functional studies

Bleeding time (Table 2) was prolonged in 12 out of 18 patients, but it never reached levels which gave cause for concern.

Platelet number was found to vary considerably but, more importantly, overall platelet function proved to be consistently (sometimes profoundly) altered. In fact, considering the aggregation parameters (Table 2), only two patients showed a normal initial latency value and only 4 had a L/50 value in the normal

Table 3. Results of immunohistochemical staining in 3 patients affected by IM. All parameters show a marked reduction in vWF-positive granules in IM platelets.

	mean ± SD		
	IM	controls	p
positive granules per μm^2	1.29±0.33	4.05±1.27	< 0.001
positive granules per platelet	4.42±0.81	5.55±0.63	< 0.001
colloidal gold particles per positive granules	5.58±1.14	8.17±1.38	< 0.001
colloidal gold particles per platelet	24.54±9.21	45.8±12.9	< 0.001

range after collagen was added. Eleven out of 18 cases showed a reduction in maximum width (Table 2). Platelet aggregation following addition of ADP and adrenalin was also found to be severely impaired in most patients, whereas ristocetin agglutination was always normal or increased.

Plasma β-TG and PF4 levels were frequently found in excess of the normal range.

In conclusion, platelet function proved to be severely impaired with regard to all the parameters studied.

Immunocytochemistry

The immunogold particles, which demonstrated the reaction with vWF, were specifically visible in platelet α-granules from both IM patients and normal subjects (Figure 1). However, the gold particles were not evenly distributed in these granules, but rather were restricted to the periphery. Only occasionally was it possible to observe a positive reaction in the SCS cisternae and on the platelet membrane (Figure 1).

In 3 myelofibrotic patients (Table 3), the number of positive α-granules per platelet and per μm^2 was markedly reduced ($p<0.001$) compared to normal controls. Moreover, the reduction in the vWF-positive reaction, as indicated

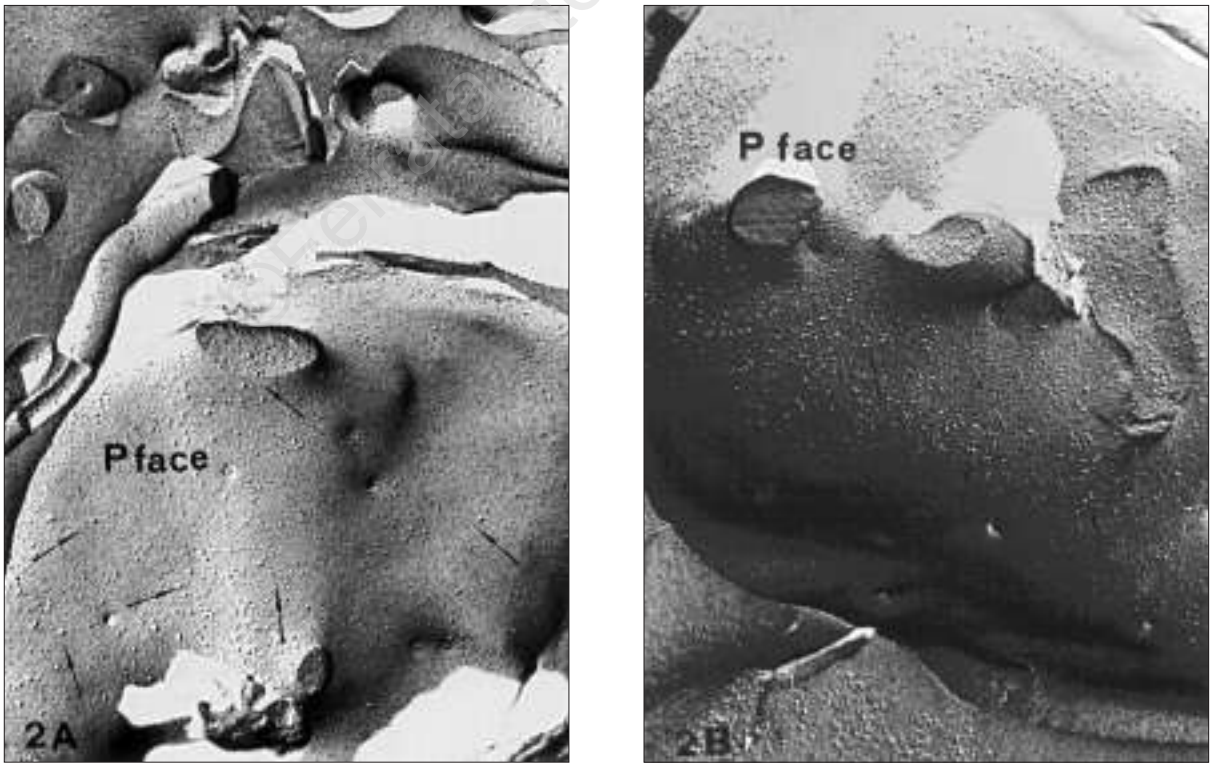

Figure 2. Intramembranous particles (⟶) on the P face of a platelet membrane from a control (A) and an IM patient (B) where there is a quantitative reduction in IMP (see text). Magnification 50,000x.

Table 4. Number, diameter and distribution of IMPs in freeze-fractured platelet membranes from 7 patients affected by IM. The IMP increase is significant only on the P face. Distribution factor (F.V.) values are not significantly different among IM patients, controls and controls plus ADP.

	P face			*E face*		
patients	n°/μm^2	∅	F.V.	n°/μm^2	∅	F.V.
MFI (7)	956,16 ±48.56	9.01 ±0.63	1.79 ±0.44	1272,96 ±116.64	8.74 ±0.47	2.45 ±0.49
	p < 0.05	NS	NS	NS	NS	NS
Controls + ADP (3)	786.24 ±31.68	9.72 ±0.34	1.95 ±0.7	1216.8 ±146.88	9.64 ±0.19	2.49 ±0.17
	NS	NS	NS	NS	NS	NS
Controls (3)	816.48 ±148.3	9.98 ±0.29	2.16 ±0.81	1225.44 ±119.52	9.45 ±0.64	3.35 ±0.56

by the number of immunogold particles per α-granule, was always statistically significant ($p< 0.001$) in patient platelets. Naturally, the total number of gold particles per single platelet was statistically lower in IM subjects than in control platelets.

Freeze fracture

The ultrastructural images of freeze-fractured platelets from IM patients and controls show the fracturing plane sectioning the phospholipid bilayer of the plasma membrane on the internal or protoplasmatic face (P face) appearing in a convex pattern (Figure 2), and on the external or exoplasmatic face (E face) (not shown), in a concave pattern.

In the 7 patients studied (Table 4), the mean IMP number per square micrometer on the P face was greater than in control subjects, although the difference proved to be significant ($p < 0.05$) only in comparison with normal platelets preincubated with ADP (which, however, never induced any visible aggregation).

The IMP diameter on the P face was substantially unchanged in myelofibrotic platelets.

No differences in IMP number or diameter were observed on the E face between patient and control platelets.

In order to obtain accurate data on IMP aggregation, we used the V statistic factor (FAC-V, as described in materials and methods), a sensitive quantitative assay for analysis of particle distribution in random, regular models or low levels of aggregation not readily distinguishable on visual inspection.

Table 4 shows calculations of IMP distribution (FAC-V) for each group of patients and controls on the P and E faces. IMPs on the P face are distributed in an overall random pattern (or at least one of low aggregation) in both patients and controls. In these cases, in fact, FAC-V values mainly lie between 1 and 2 (1.79±0.44 in IM patients, 2.16±0.81 in controls and 1.95±0.7 in controls plus ADP).

On the E face IMPs appear to be slightly more aggregated in IM patients, as shown by higher values of FAC-V (2.45±0.49 in IM patients and 3.35±0.56 and 2.49±0.17 in controls and controls plus ADP, respectively), but no significant difference was found among the three groups.

We compared our results to internal controls because of a certain discrepacy among some observations, particularly for IMP density. These differences may be accounted for by the relative novelty and continuous evolution of the freeze fracturing technique.

Morphology of aggregated platelets

Collagen: ultrastructural analysis showed the characteristic morphology of *in vitro* collagen-aggregated platelets, in both normal subjects and IM patients. In control platelets, whose *in vitro* aggregation was normal, the agonist agents caused almost complete platelet degran-

Table 5. Levels of free cytosolic calcium (11 patients), membrane Na^+/K^+ATPase (13 patients) and Ca^{++}ATPase (13 patients) in IM platelets. The difference between patients and normal controls (36 subjects) is strikingly significant.

	n°	IM M±SD	controls M±SD	p
intraplatelet calcium (nmol/L)	11	252.36±62	103±21	< 0.001
Na^+/K^+ ATPase (μmol Pi × mg $prot^{-1}$ × h^{-1})	13	0.53±0.36	1.32±0.09	< 0.001
Ca^{++} ATPase (μmol Pi × mg $prot^{-1}$ × 90 min^{-1})	13	0.232±0.081	0.109±0.013	< 0.001

ulation and a high degree of cellular anisocytosis, with platelets adhering to and interdigitating tightly with one other. In IM patients, the ultrastructural pattern was characterized by incomplete degranulation correlated to the degree of functional *in vitro* impairment (Figure 3).

Ristocetin: the ultrastructural image of normal ristocetin-agglutinated platelets demonstrated the presence of a variable number of cytoplasmatic granules, in striking contrast with the collagen-induced aggregation pattern. Moreover, platelets most frequently appeared to be discoid in shape.

In IM patients, too, the degranulation caused by addition of ristocetin was incomplete, but to a lesser extent, paralleling the functional tests which demonstrated a sharp trend towards normal/high values in the case of ristocetin agglutination (Figure 4).

Biochemical studies

The intraplatelet calcium level of 11 patients (Table 5) proved to be consistently higher than in control subjects; the difference between the two groups was statistically significant.

Na^+/K^+ATPase activity measurements for patients proved to be severely reduced in comparison with normal controls, while the mean level of Ca^{++} ATPase activity in patients was almost double that of normal platelets.

Discussion

Functional tests in our IM patients showed extremely severe alteration in platelet aggregation in response to ADP, epinephrine and collagen, as previously described.[9] On the other hand, platelet agglutination in response to ristocetin was always normal or elevated, which is in accordance with our electron microscope findings of marked platelet degranulation after adding ristocetin.

Recently, the pathogenesis of IM has been related to defective megakaryocyte and platelet functions (aggregation and release) that lead to an inappropriate release of α-granule content, including vWF and growth factors (megakaryocyte-derived growth factor and PDGF).[27-30]

A reduction of vWF in platelet α-granules from IM patients was also demonstrated by our immunogold assay, and is in agreement with previous studies which used electron-immunoassay measurements to document intraplatelet depletion of vWF.[8] Furthermore, lack of active vWF in the *exhausted* platelets might play an important role in the pathogenesis of hemorrhages, which are frequently described in IM patients; however, we never observed any clinical alteration of hemostasis in the study group.

Both the deficiency of intraplatelet vWF (normally equal to 10% of the total circulating vWF pool) and the increased plasma levels of β-TG and PF4 seem to be the result of increased release.[3, 31]

This is really a crucial point; however, the possibility our results may be the expression of *in vitro* activation due to preparation of the test sample is quite improbable.

As a matter of fact, first, we tried to avoid any pre-analytical pitfalls by strictly controlling blood drawing and sample preparation; second, all analytical results were fitted in a multiple point calibration curve; third, if *in vitro* activation had taken place, we would have expected to observe even higher values, especially of PF-

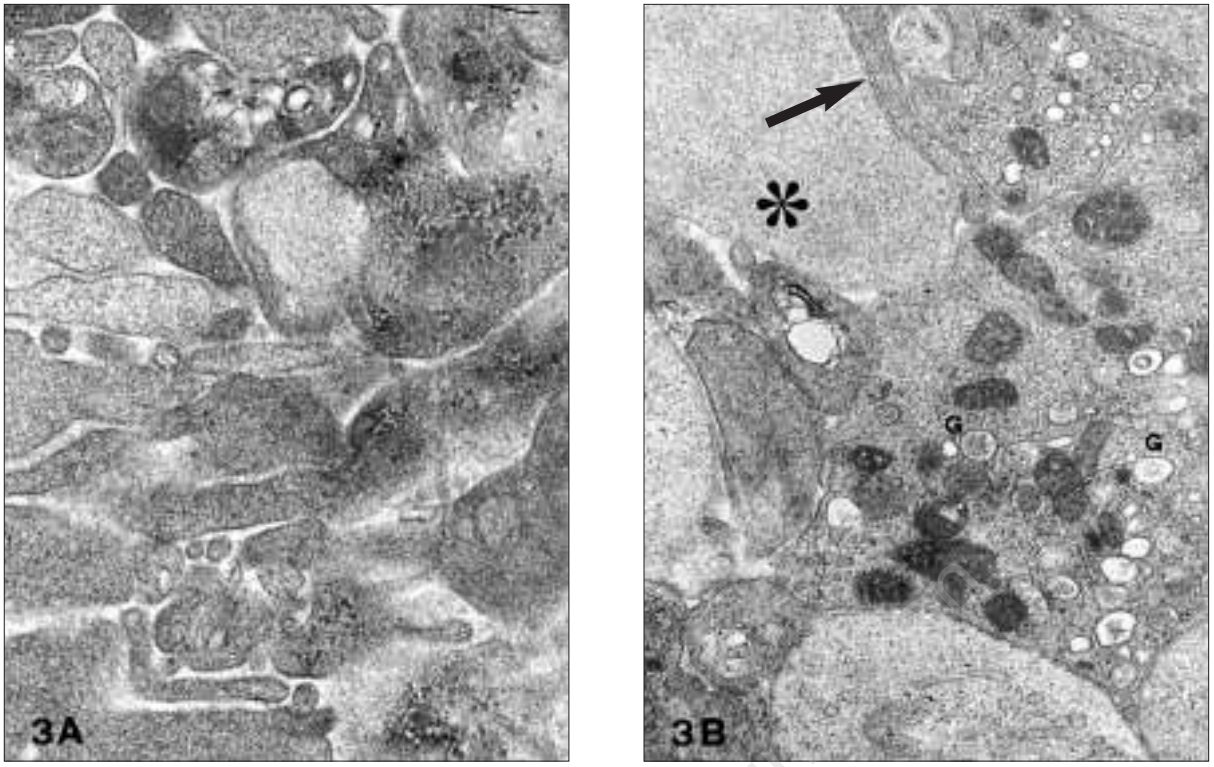

Figure 3. Platelet aggregates from a control (A) (30,000x) and an IM patient (B) (32,000x) after addition of collagen. All normal platelets appear to be almost completely deprived of granules (A). Conversely, the degranulation of IM platelets is strikingly less evident (B) (i), and even in the presence of an evident shape change (→) some granules can still be seen (**G**).

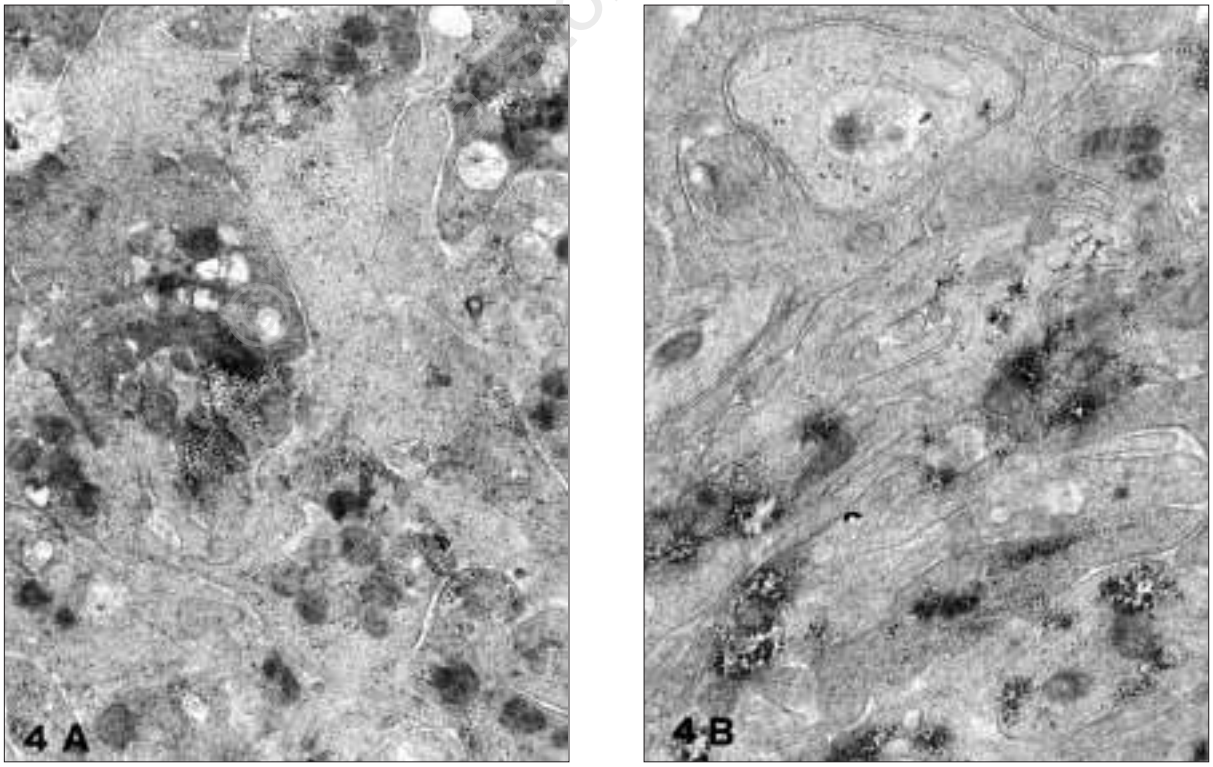

Figure 4. Ristocetin-induced platelet agglutination in a control (A) (22,000x) and an IM patient (B) (25,000x). After addition of ristocetin, more evident granule depletion can be observed in IM than in normal platelets (A), which sometimes appear to be full of granules and to have less marked membrane interconnections.

4, with an alteration of the β-TG/PF-4 ratio (normally 4-5:1). Actually our results, although frequently elevated, are not that far above normal and, lastly, have been confirmed by previous studies.[14]

The elevated intraplatelet Ca^{++} content in IM also suggests an increase in platelet *activation*.[32] Platelet membrane Na^+/K^+ ATPase and Ca^{++} ATPase are both altered in IM patients with respect to controls.

The reduction in Na^+/K^+ATPase activity might be explained either by primary abnormalities of the platelet membrane or by a decrease in platelet energy reserves, which would deplete ATP levels. The lower Na^+/K^+ ATPase activity might also determine, in part, an increase in intraplatelet Ca^+ through elevation of intracellular Na^+ and secondary activation of plasma membrane Na^+/Ca^+ exchange.[33] It would also be possible for the platelets in IM to become activated through the action of circulating factors such as immune complexes, frequently described in this disease, or by passing through medullary and/or splenic capillaries and sinuses altered by fibrosis and/or neoplastic invasion. Furthermore, the anomalous release of PDGF (a fibroblast mitogenic factor), PF-4 (a collagenase inhibitor) and TGF-β (a fibrogenetic factor) from platelets and abnormal megakaryocytes almost surely plays a pathogenetic role in the development of fibrosis.[34]

Nevertheless, the degree of platelet structural alteration in IM is still difficult to evaluate, and our freeze-fracturing investigations showed no features of morphological abnormality at the IM plasma membrane level. In fact, IMPs were only slightly increased on the P face of IM platelets as compared with control platelets activated by ADP. IMPs are generally considered to be membrane protein molecules, and their number and distribution seem to be related to membrane biochemical functions.[35]

IMP density and distribution have already been studied in pathologies caused by platelet dysfunction, such as Glanzmann's thrombasthenia and the Bernard-Soulier syndrome. Such diseases present alterations in the membrane glycoproteins involved in the aggregation and adhesion processes. An overall reduction in IMPs can be observed in Glanzmann thromboasthenia.[36] This finding can also be noted in the Bernard-Soulier syndrome, where it is associated with a higher relative numerical density of IMPs on the P than on the E faces. An increase in IMP density on P faces has also been demonstrated in platelet membranes obtained during clot retraction in healthy individuals, but activation and aggregation by serum or ADP do not modify the density or distribution of IMPs in normal platelets.[37, 38]

The higher free cytosolic calcium concentration found in our patient platelets might be responsible for the increase in number of IMPs on the P face of the platelet membranes of IM patients. In fact, our previous study[39] demonstrated that *in vitro* addition of calcium to isolated cell membranes modifies the number of IMPs on both P and E faces.

In conclusion, our data confirm severe platelet morphofunctional abnormality in IM, suggesting that these cells play an intriguing role in the pathogenesis of IM. At present, it seems possible to sustain that, as in other CMDs, IM platelets present structural abnormalities caused primarily by a malformative differentiation process. As a consequence, IM platelet activation might also be abnormally and more easily triggered thanks to the anomalous fibrotic microenvironment induced by this pathology.

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